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Award Number: DAMD17-98-1-8590

TITLE: Signal Transduction Pathway in Maspin-induced Tumor Suppression of Prostate Cancer

PRINCIPAL INVESTIGATOR: Karl X. Chai, Ph.D.

CONTRACTING ORGANIZATION: University of Central Florida
Orlando, Florida 32816

REPORT DATE: September 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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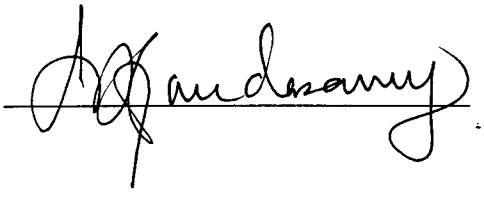

12/21/00

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1999	Annual (1 Sep 98 - 31 Aug 99)

4. TITLE AND SUBTITLE
Signal Transduction Pathway in Maspin-induced Tumor Suppression of Prostate Cancer

5. FUNDING NUMBERS
DAMD17-98-1-8590

6. AUTHOR(S)
Karl X. Chai, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Department of Molecular Biology and Microbiology
University of Central Florida, 4000 Central Florida Boulevard
Orlando, Florida 32816
E-MAIL:
kxchai@pegasus.cc.ucf.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
This report contains colored photos

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13. ABSTRACT *(Maximum 200 Words)*

The purpose of this project was to identify a maspin receptor, and to investigate the signal transduction in maspin-induced cancer suppression. Human prostasin was examined as a candidate for maspin receptor. Our major progress is in the following areas. First, prostasin expression is found down-regulated in prostate cancer. Second, PC-3 cells transfected with a human prostasin cDNA displayed a significantly reduced level of invasiveness (50-80% inhibition). Third, the prostasin-transfected PC-3 cells have an increased maspin expression, suggesting a concerted tumor suppression mechanism between these two proteins. Fourth, prostasin expression in LNCaP cells can be up-regulated by dihydrotestosterone, suggesting a possibility that prostasin's down-regulation in prostate cancer is not due to gene damage. Fifth, a human maspin-expressing fruit-fly strain has been established, as well as a strain that expresses human hepsin, a second candidate for maspin receptor. Overall, we have tested the initial hypothesis at or exceeding the pace intended. Several unexpected findings were made regarding the behaviors of human prostasin, and the stage is set for the follow-through investigation on how maspin and prostasin work synergistically to suppress prostate cancer. A prostate cancer therapy and methods of diagnosis are being developed as part of the extended goal of our research.

14. SUBJECT TERMS			15. NUMBER OF PAGES
Prostate Cancer, maspin, receptor, prostasin, Drosophila, mutation			18
			16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Limited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

[redacted]

FOREWORD

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Sept. 29, 1999

PI - Signature Date

(4). TABLE OF CONTENTS:

(5) INTRODUCTION:

Maspin is a serine protease inhibitor that suppresses breast and prostate cancers. The investigation of this tumor suppression in breast and prostate cancer cells led to the speculation of maspin's interaction with a membrane-bound serine protease in these tissues or cancers. This interaction may be an initial signaling event to the tumor suppression pathways (manifested as inhibition of tumor cells' motility and invasiveness in *in vitro* and *in vivo* assays) (Sheng *et al.*, 1996). We had suggested in our original proposal that a human prostate-produced serine protease, prostasin, can serve the role of a maspin receptor/interactive protease based on our evaluation of prostasin's putative structure and its substrate preference (Yu *et al.*, 1994). We intended to investigate whether a direct interaction between maspin and prostasin can be established using conventional biochemistry and molecular biology methods. We also intended to identify the down-stream proteins in the maspin/prostasin signal transduction pathway taking advantage of *Drosophila* genetics methods. Further, an alternative candidate protease, human hepsin, will also be investigated for the same functions.

(6) BODY:

For the funding period of September 1, 1998 to August 31, 1999, the tasks outlined in the approved Statement of Work were

a). perform binding of radiolabeled recombinant maspin to the cell membrane of LNCaP cells (months 1-6)

b). perform interference of maspin binding to LNCaP cells by the serine protease inhibitor aprotinin (months 7-9)

c). perform binding of labeled maspin to human 293 cells transfected with a prostasin cDNA (months 10-12)

d). clone and characterize *Drosophila* homologues of maspin and prostasin (months 1-6)

e). initiate mutagenesis to isolate mutations in d-maspin and d-prostasin (months 7-12)

When funding was approved for our project, we also received several excellent suggestions from the reviewers. The major suggestions are:

a). Regarding the choice of prostate cancer cell line for our studies, the LNCaP cells, reviewer A suggested "the biological behavior of lymph node cancer of prostate origin (LNCaP) cells should be verified before a high-risk project that studies the invasion properties of these cells is undertaken". Reviewer B's comments were "the use of LNCaP cells for studies to identify the receptor for maspin may not be appropriate. LNCaP cells are known to be the least invasive cells among the commonly used human CaP cell lines (*e.g.*, PC-3, DU-145, LNCaP). If a protease and its inhibitor and the inhibition of invasion by maspin are to be studied, a cell line that shows considerable invasion should be used so that inhibition of invasion by the test agent could be measured".

b). Regarding the initiation of the *Drosophila* portion of the project, reviewer A's comments were "The *Drosophila* experiments are exciting and there is a good rationale for utilizing this approach. However, strategies to identify maspin-prostasin interactions in human cells should be more thoroughly exhausted first. The *Drosophila* genetics will undoubtedly provide important new information, but human cell data will be more relevant to the CaP problem". From reviewer B, the comments were "At present, very little is known about prostasin and maspin in the prostate. It would be logical first to establish their natural occurrence and interaction; then a study of the genes

.in *Drosophila* may be appropriate. However, what the function of prostasin is, what its natural substrates are, and if maspin is a naturally occurring serpin in the prostate are questions that need to be answered first".

Research Progress:

1). Prostasin and maspin interaction in human prostate cancer:

Based on these excellent suggestions to our proposal, we had decided at the onset of our work to examine the pair of our target proteins in a more invasive prostate cancer cell line, the PC-3. Using techniques such as northern blot hybridization, RT-PCR, and western blot analysis, we were unable to detect the expression of human prostasin in this cell line (Figures 1 and 4, Appendix 1). On the other hand, expression of prostasin can be detected in the LNCaP cells (Figure 2, Appendix 1). The original findings by Sheng *et al.* (1996) had clearly demonstrated maspin's effectiveness in suppressing the PC-3 cell line, at this moment, the hypothesis that prostasin is a receptor for maspin must be reconsidered. Prostasin may still have the ability to interact with maspin because the protease prefers a substrate bond which is identical to maspin's reactive site bond (RSB); but an interaction between prostasin and maspin is not necessarily a prelude to the maspin signaling pathway. As a result, we did not follow-through with the binding assays mentioned above (using the LNCaP and prostasin-transfected 293 cells). Since the PC-3 is a progressively worse cancer than the LNCaP, and we also know that normal prostates produce a large amount of prostasin (Yu *et al.,* 1994), we speculated that prostasin may actually be down-regulated in prostate cancer. This protease may be a part of normal instead of cancerous prostate physiology. With a grant from the Florida Hospital Gala Endowed Program for Oncologic Research, we recruited human CaP specimens and performed immunohistochemistry studies on the slides. The results show that normal sections of the prostate display a high level of prostasin protein expression in the ductal epithelial cells (also see Yu *et al.,* 1994); while the cancerous sections show little or none expression in either the cancer itself, or the epithelial cells lining the duct (Figure 3, Appendix 1).

The next experiment we performed was to introduce prostasin protease back into the PC-3 cells *via* recombinant DNA and to evaluate the behaviors of the transfectants. A full-length human prostasin cDNA (Yu *et al.,* 1995) was subcloned into the pcDNA3 vector (Invitrogen, Carlsbad, CA) and transfected into PC-3 cells *via* electroporation. G418 selection was performed to establish 4 transfectants expressing prostasin (named PCPC-104, -105, -107, and -1015) and 4 transfectants that contained the vector control (named PCPC-101, -108, -1013, and -1021) (Figure 4, Panel A, Appendix 1, not all transfectants are shown).

In an *in vitro* Matrigel invasion assay (method adopted from Liu and Rabbani, 1995), we evaluated the invasiveness of the prostasin-expressing PC-3 cells. Figure 5 (Appendix 1) shows the comparison of the number of cells that invaded through the Matrigel barrier between the parent cell line, the prostasin-expressing transfectants, and a vector control transfectant.

As a test for our original hypothesis that maspin and prostasin interact with each other (but may not necessarily do so directly since the PC-3 cell line does not express prostasin), and as a test to see if the invasion inhibition by maspin and that by prostasin utilize overlapping signal transduction pathways; we examined the prostasin-expressing PC-3 cells for maspin expression. As a result, we have shown clearly that maspin's expression is induced by the introduction of prostasin in PC-3 cells (Figure 4, Panel B, Appendix 1).

Once we had realized that prostasin's probable cancer biology is its down-regulation with the progression of tumor, we attempted to answer whether the down-regulation is the result of gene

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(DNA) damages. We examined the prostasin gene expression in LNCaP cells in response to hormone. Figure 2 (Appendix 1) shows a western blot analysis of LNCaP cells treated with dihydrotestosterone (DHT). Prostasin protein expression is seen increased by either duration or dosage of treatment. At this point, we have not tested whether the prostasin gene has any DNA damages in the PC-3 cells. Since the PC-3 cell line is not hormonally responsive, a different method to potentially induce the prostasin gene expression in this cancer cell line needs to be developed.

2). Maspin, hepsin, and prostasin transgenic flies for the elucidation of the tumor suppression signal pathway(s):

A major event in the early part of the past funding year led us to the decision of creating transgenic flies that carry functional human genes in the hypothetical maspin signal pathway, namely, the genes coding for maspin, hepsin, and prostasin. These genes are being misexpressed in the *Drosophila* eye, an organ that is non-essential for survival. The major event that led to this new strategy is the near and eventual completion of the *Drosophila* genome project, followed by the availability of all relevant sequence data to the scientific community. Upon a thorough interrogation of the *Drosophila* genome database, we had realized that there could potentially be several candidate genes for either the maspin or the prostasin homologue in flies (Table 1, Appendix 1). All of these candidate genes share an equally extensive sequence homology with the human target genes that it is believed that a conventional library screening would have picked up all. In our library screening efforts prior to the availability of the *Drosophila* sequence data, we have experienced a high number of positive signals for the human prostasin cDNA probe. Such an observation can now be explained by the high number of potential homologue genes. Without any information regarding the function or even the expression pattern of these potential candidate genes, identification of the true homologues of human maspin or prostasin would have been a daunting task. The collaborating laboratories, therefore, decided to change the strategy of taking advantages of *Drosophila* genetics, from using native *Drosophila* genes, to using human transgenes. If our original idea of two-way cross-examination of protein function is still valid, using human transgenes in flies will still produce the potential phenotypes that can lead to the identification of proteins that interact with the subject proteins under study.

Another major factor in our decision to switch our strategy from cloning *Drosophila* homologues of human proteases and serpins to misexpression of these genes in the *Drosophila* eye was an abundance of recently published evidence indicating that this strategy is both biologically reasonable and feasible (reviewed in Thomas and Wassarman, 1999). Current estimates suggest that two thirds of all vital *Drosophila* genes are required for eye development. This is not surprising because the eye is a complex organ containing many cell types, and develops *via* a complex interacting network of genes coordinating cell-cycle and cell-death pathways with cellular differentiation programs. Many *Drosophila* signal transduction pathways utilized during eye development have been characterized. Based on experience, foreign genes misexpressed in the *Drosophila* eye interfere with eye development at a high frequency (approximately 50%). Most likely, these genes interact negatively with existing signaling pathways. In some cases an eye phenotype generated by misexpression of a human gene disrupts a process controlled by known signaling pathways. Given the remarkable degree of conservation of these pathways between *Drosophila* and higher vertebrates, any information concerning the involvement of maspin, prostasin, or hepsin in these pathways is immediately transferable to human cell culture experimental systems.

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Transgenic flies misexpressing hepsin and maspin in the *Drosophila* eye have been generated. Two independent insertions of the hepsin transgene into the *Drosophila* genome have been generated. The human hepsin transgene is under the control of the *Drosophila glass*-promoter, which directs eye-specific expression. Both transgenic hepsin strains exhibit severe eye abnormalities. A maspin transgene under the control of the *Drosophila eyeless*-promoter has been inserted into 7 independent locations in the *Drosophila* genome. None of the transgenic lines carrying the maspin gene have an eye phenotype. Although the maspin transgenic flies do not generate a phenotype, they are still a valuable reagent. By crossing flies carrying the maspin transgene to flies carrying the hepsin transgene, we can ask if co-expression of maspin and hepsin in the eye modifies the hepsin phenotype. If the hepsin phenotype is modified, we will have evidence for an *in vivo* interaction (not necessarily direct) between maspin and hepsin. (Efforts are currently underway to create hepsin- and maspin-transgenic flies with matching promoters for crossing.)

We are currently also in the process of constructing transgenic flies misexpressing prostasin in the eye (again, using the *glass*- and the *eyeless*-promoters). If these transgenic flies have an eye phenotype, we will co-express maspin and prostasin in the eye to determine if these proteins interact *in vivo*.

(7) KEY RESEARCH ACCOMPLISHMENTS:

____ Prostasin expression is found to be down-regulated in cancer versus normal control in the prostate.

____ Human prostate cancer bone metastasis cell line PC-3 transfected with a full-length human prostasin cDNA displayed a significantly reduced level of invasiveness (50-80% inhibition) in an *in vitro* Matrigel invasion assay.

____ The transfected PC-3 cells expressing human prostasin also have an increased expression of human maspin, suggesting a concerted tumor suppression mechanism between these two proteins.

____ Prostasin expression in human prostate cancer lymph node metastasis cell line LNCaP can be up-regulated by treatment with dihydrotestosterone (DHT), suggesting a possibility that prostasin's down-regulation in prostate cancer is not a result of gene (DNA) damage.

____ A human maspin-expressing transgenic fruit-fly strain has been established in the Co-PI's laboratory, as well as a transgenic strain that expresses human hepsin serine protease, a second candidate for maspin receptor/interactive protease.

(8) REPORTABLE OUTCOMES:

____ Human prostate cancer bone metastasis cell line PC-3 transfected with a full-length human prostasin cDNA expresses human prostasin at the mRNA and the protein levels, and have a apparently lesser degree of invasiveness. Several prostasin-expressing transfectants are established, as well as several vector control transfectants.

(9) CONCLUSIONS:

The currently completed research tasks are considered on schedule with regard to the approved Statement of Work. We have found that human prostasin is a potential suppressor of prostate cancer and that its tumor suppression pathway may be overlapping with the maspin tumor suppression

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pathway. Several intended tasks for the first year were not performed due to our new findings, but the tasks planned for the ensuing years remain highly appropriate. In addition, several new tasks are being contemplated (see below in the "so what section" for details), pending the availability of future funding.

"So What Section" The currently completed research has the following implications toward the diagnosis or treatment of human prostate cancer:

1). An assay (test) may be established to determine the level of production of human prostasin in CaP either using an immunological reagent (antibody) or using a nucleic acid reagent (part of prostasin's gene). The results may be used in diagnosis or prognosis of the invasiveness of the human CaP.

2). An immunological assay (test) may also be established to determine the level of human prostasin in bodily fluids such as (but not limited to) blood, semen, or urine. The results may be used to aid the diagnosis of cancer of various grades, pending continued research to demonstrate the correlation between levels of prostasin in various bodily fluids and the presence of cancer.

3). Purified whole human prostasin may be used as a deliverable drug to patients with a CaP in which the *in situ* production of prostasin is determined to be reduced or deficient either by an immunological or a nucleic acid (RNA) assay (test).

4). Purified partial human prostasin may also be used in an application as that described above in 3)., pending continued research to demonstrate the effectiveness of a partial human prostasin in inhibiting cancer invasion.

5). A human prostasin gene (cDNA) may be incorporated into a gene therapy vector and used for treating invasive CaP in which the *in situ* production of prostasin is determined to be reduced or deficient.

6). If a compound is found to be able to induce the production of prostasin from invasive CaP in which the *in situ* production of prostasin is determined to be reduced or deficient, this compound may be used as a deliverable drug to patients with such CaP; pending continued research to demonstrate that the reduction or deficiency of prostasin production in these CaP is not a result of gene damage at the human prostasin genetic locus.

[redacted]

(10) REFERENCES:

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Sheng, S., Carey, J., Seftor, E. A., Dias, L., Hendrix, M. J. C., and Sager, R. (1996) Maspin acts at the cell membrane to inhibit invasion and motility of mammary and prostatic cancer cells. ***Proc. Natl. Acad. Sci. USA*** 93: 11669-11674.

Thomas, B. J., and Wassarman, D. A. (1999) A fly's eye view of biology. ***TIG*** 15: 184-190.

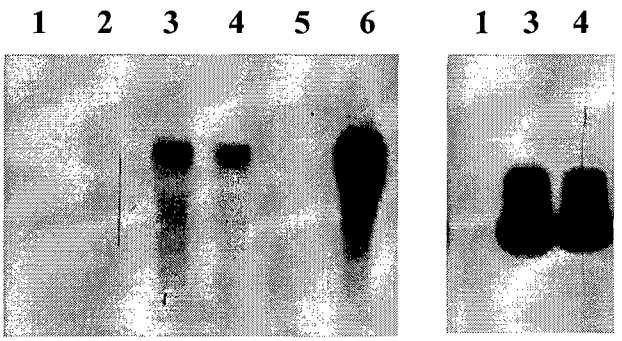
Yu, J. X., Chao, L., and Chao, J. (1994) Prostasin is a novel human serine protease from seminal fluid. ***J. Biol. Chem.*** 269: 18843-18848.

Yu, J. X., Chao, L., and Chao, J. (1995) Molecular cloning, tissue-specific expression, and cellular localization of human prostasin mRNA. ***J. Biol. Chem.*** 270: 13483-13489.

(11) APPENDICES:

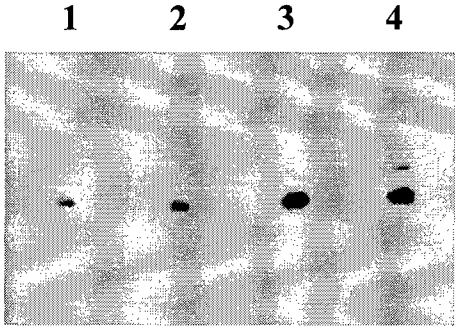
Appendix 1. Figures and Tables

Figure 1. Northern blot analysis and RT-PCR/Southern blot analysis of prostasin expression. Lanes: 1, PC-3; 2, PCPC-101; 3, PCPC-105; 4, PCPC-107; 5, 293 cells; 6, 293 cells transfected with a pREP-8/Human prostasin cDNA construct. Total RNA was isolated from the various cells using the QIAGEN RNeasy kit. For the northern blot analysis, 20 μg of total RNA were loaded into each lane. The northern blot was hybridized to a nick-translated human prostasin cDNA probe and washed to a final stringency of 1 x SSPE at 65°C before exposure at -80°C for 5 days with an intensifying screen. For the RT-PCR/Southern blot analysis, 1 μg of total RNA was used in the RT-PCR using two human prostasin gene-specific oligonucleotide primers, the Southern blot of the resolved RT-PCR bands was probed with a third gene-specific oligonucleotide as described (Yu *et al.,* 1995).



Northern Blot Analysis **RT-PCR**

Figure 2. Western blot analysis showing a DHT induction of prostasin expression in LNCaP cells. Lanes: 1, LNCaP, serum starved in serum-free medium for 24 hours; 2, LNCaP, serum-released for 24 hours in regular culture medium (RPMI-1640) containing 10% FBS; 3, LNCaP, in regular culture medium supplemented with 100 nM DHT for 24 hours; 4, LNCaP, in regular culture medium supplemented with 100 nM DHT for 48 hours. Cells were harvested by mechanical force and washed 3 times in 1 x PBS (pH7.2) before being lysed for protein concentration determination using a Bio-Rad DC protein assay kit. An equal amount of protein (80 μg) was loaded in each lane for the SDS-PAGE. The resolved proteins were electro-transferred to a nitrocellulose membrane. A 1:1,000 diluted rabbit anti-human prostasin antibody was used for the western blot analysis. The Pierce WestPico enhanced chemilluminescence reagent was used to detect the signals *via* a goat anti-rabbit IgG secondary antibody coupled to horse radish peroxidase (used at a 1:10,000 dilution).



DHT Induction of Prostasin Expression in LNCaP Cells

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Figure 3. Immunohistochemistry staining of normal and cancer sections of human prostate to detect the expression of human prostasin. Paraffin-embedded human prostate sections (4-μm) were de-paraffinized in xylene followed by rehydration in a decreasing series of alcohol. The endogenous peroxidase activity was inhibited by incubating the sections with H_2O_2. After blocking the sections with Tris-buffered saline containing 0.1% Tween-20 (TBST) and 10% goat serum, the prostasin-specific rabbit antiserum (diluted 1:500 in the blocking solution) was added for an incubation of 2 hours followed by an incubation of 30 minutes with the secondary antibody, goat anti-rabbit IgG (1:50). The sections were then incubated with peroxidase anti-peroxidase complex (1:200) for 30 minutes and they were washed in TBST between steps. The color reaction was performed by incubating the prostate sections with 3,3'-diaminobenzidine tetrahydrocholoride dihydrate and H_2O_2. The prostate sections were counter-stained with hemotoxylin, dehydrated in an increasing series of alcohol and mounted with *Permount*. The sections were then viewed and photo-captured under light microscope. The brown staining can be seen in the epithelial cells of the secretory ducts of the prostate as well as the prostatic fluid inside the lumen indicating that prostasin is expressed and secreted by the epithelial cells of the prostate (left panel). None or light staining can be seen in the tumor mass of the prostate epithelial cells (right panel).

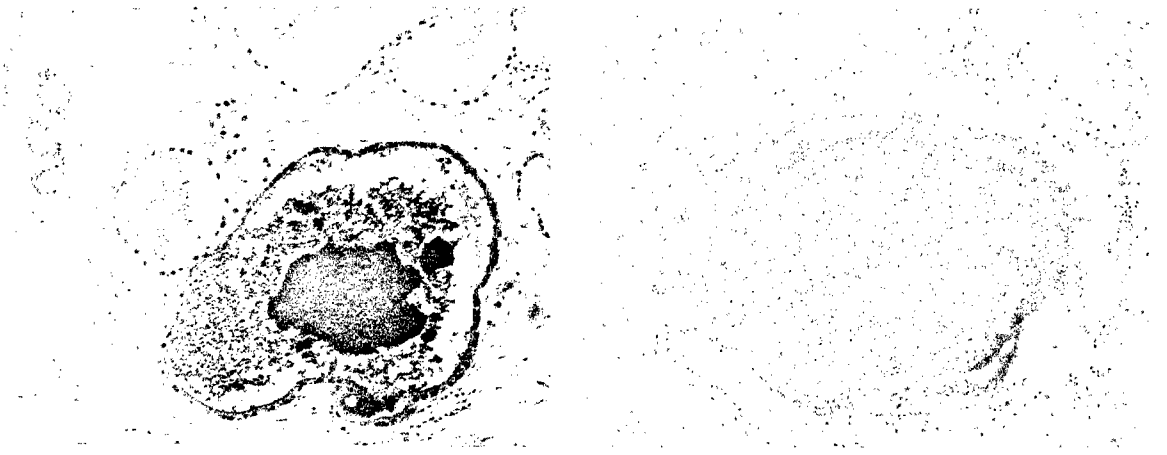

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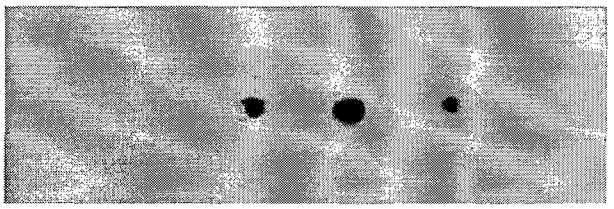
Figure 4. Western blot analysis of the expression of human prostasin and maspin in PC-3 cells transfected with human prostasin cDNA and pcDNA3 vector. Lanes: 1, PC-3, 2-6: PC-3 transfectants: PCPC-101, PCPC-104, PCPC-105, PCPC-107, and PCPC-108. Cells were cultured in F-12K/FBS (9:1) medium with G418 (400 μg/ml) when appropriate to 50% confluence, washed in 1xPBS, and harvested with a scraper. An equal amount of protein (35 μg) was loaded in each lane for the SDS-PAGE. The western blot procedures are as described in the legend to Figure 2.

A: Western Blot with Prostasin Antibody



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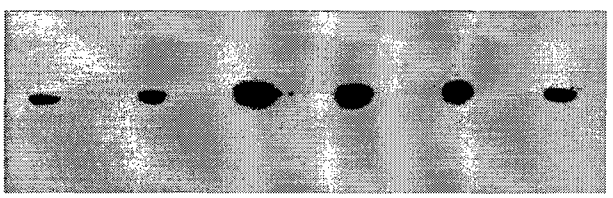

B: Western Blot with Maspin Antibody

Figure 5. *In vitro* Matrigel invasion assays using PC-3 cells and PC-3 transfectants expressing human prostasin. Cell types: clockwise from the upper left panel, PC-3, PCPC-101, PCPC-105, PCPC-107. Cells were cultured in F-12K/FBS (9:1) medium with G418 (400 μg/ml) when appropriate to 80% confluence before being harvested *via* trypsinization. Cells were then washed, counted, and resuspended in serum-free medium. Transwell inserts (growth area: 0.33 cm^2) with 8-μm pores were coated with 50 μg of Matrigel (Collaborative Biochemical) and solidified at 37°C (in a moist chamber) for 60 minutes before use. Assays were set up in triplicates according to the procedures described by Liu and Rabbani (1995), each well receiving 5,000 cells. The invasion process was allowed to proceed for 24 hours in a CO_2 incubator. The cells were then washed, fixed, and stained with toluidine blue. Cells on the surface of the Matrigel membrane were removed with a Q-tip, and the cells that invaded through were counted or photo-captured.

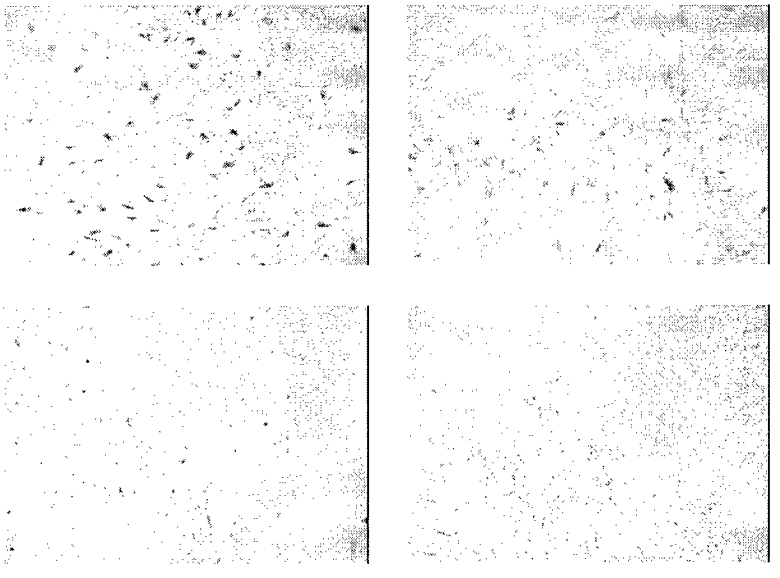

Table I. Candidate *Drosophila* homologues of human maspin and prostasin.

Fly Gene Accession Number	*Percent Homology with Human Maspin	Prostasin
AC005556		38
AC004290		35
AC005653		31-48**
LP06382		33
AC005814		29-60**
AC005638		31-45**
AC006495		31
LP06207		34-36**
AC004336	31-36**	
AC004532	29-55**	
AC005834	29-42**	
AC008340	25-50**	
AC005860	25-46**	
AC004301	27	
AC006469	23-46**	
AC007549	22-50**	
AC007467	30	

*Percentage homology is determined from amino acid sequence alignment, counting the identical amino acid residues shared between two (segments of) sequences.

**Multiple discrete regions are aligned between the human and the fly sequences, with various levels of degree of homology.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 28 July 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
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